(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
14 December 2000 (14.12.2000)

PCT

(10) International Publication Number
WO 00/75162 A1

(51) International Patent Classification[7]: C07H 21/02, 21/04, C07K 1/00, A01N 37/18, 43/04

(21) International Application Number: PCT/US00/15652

(22) International Filing Date: 7 June 2000 (07.06.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/137,859 7 June 1999 (07.06.1999) US

(71) Applicant: MIRUS CORPORATION [US/US]; 545 Science Drive, Madison, WI 53711 (US).

(72) Inventor: WOLFF, Jon, A.; 1122 University Bay Drive, Madison, WI 53705 (US).

(74) Agent: JOHNSON, Mark, K.; P.O. Box 510644, New Berlin, WI 53151-0644 (US).

(81) Designated State *(national)*: JP.

(84) Designated States *(regional)*: European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published:
— *With international search report.*
— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 00/75162 A1

(54) Title: A COMPOUND CONTAINING A LABILE DISULFIDE BOND

(57) Abstract: A labile disulfide-containing compound under physiological conditions, comprising: the disulfide-containing compound having a labile disulfide bond that is either a disulfide bond that is cleaved more rapidly than oxidized glutathione or a disulfide bond constructed from thiols in which one of the constituent thiols has a lower pKa than glutathione or a disulfide bond that is activated by intramolecular attack from a free thiol.

A Compound Containing a Labile Disulfide Bond

Background

Bifunctional molecules, commonly referred to as crosslinkers, are used to connect two molecules together. Bifunctional molecules can contain homo or heterobifunctionality. The disulfide linkage (RSSR') may be used within bifunctional molecules. The reversibility of disulfide bond formation makes them useful tools for the transient attachment of two molecules. Disulfides have been used to attach a bioactive compound and another compound (Thorpe, P.E. *J. Natl. Cancer Inst.* **1987**, *79*, 1101). The disulfide bond is reduced thereby releasing the bioactive compound. Disulfide bonds may also be used in the formation of polymers (Kishore, K., Ganesh, K. in Advances in Polymer Science, Vol. 21, Saegusa, T. Ed., 1993).

There are many commercially available reagents for the linkage of two molecules by a disulfide bond. Additionally there are bifunctional reagents that have a disulfide bond present. Typically, these reagents are based on 3-mercaptopropionic acid, i.e. dithiobispropionate. However, the rate at which these bonds are broken under physiological conditions is slow. For example, the half life of a disulfide derived from dithiobispropionimidate, an analog of 3-mercaptopropionic acid, is 27 hours in vivo (Arpicco, S., Dosio, F., Brusa, P., Crosasso, P., Cattel, L. *Bioconjugate Chem.* **1997**, *8*, 327.). A stable disulfide bond is often desirable, for example when purification of linked molecules or long circulation *in vivo* is needed. For this reason, attempts have been made to make the disulfide less susceptible to cleavage.

It has been demonstrated that both stability, measured as reduction potential, and rate, measured as rate constants, of disulfide reduction are both related to the acidity of the thiols which constitute the disulfide. Additional factors that may affect the rate of reduction are steric interactions, and intramolecular disulfide cleavage. Looking at the difference in the rates for the reactions RSH + R'SSR' → RSSR' + R'SH and RSH + R''SSR'' → RSSR'' + R''SH, it has been demonstrated that $\log k''/k' = \beta(pK_a^{R'} - pK_a^{R''})$, where k' and k'' are the rate constant for the reactions with R'SSR'

and R''SSR'' respectively, $pK_a^{R'}$ and $pK_a^{R''}$ are the acidities of the thiol groups R'SH and R''SH, and β is a constant determined empirically to be 0.72. From this equation, one would predict that the reduction of a disulfide composed from relatively acidic thiols would be reduced more quickly than one composed of less acidic thiols. In support of this observation, it has been demonstrated that the disulfides cystine (pK_a 8.3) and cystamine (pK_a 8.2) are reduced 3-15 times faster than oxidized glutathione (pK_a 8.9) (Bulaj, G., Kortemme, T., Goldenberg, D.P. *Biochemistry* **1998**, *37*, 8965).

It has been demonstrated that both stability (thermodynamics), measured as reduction potential (Keire D.A. *J. Org. Chem.* **1992**, *57*, 123), and rate (kinetics), measured as rate constants, of disulfide reduction are both related to the acidity of the thiols which constitute the disulfide (Szajewski, R.P., Whitesides, G.M. *J. Am. Chem. Soc.* **1980**, *102*, 2011).The increase in acidity of a thiol is dependent upon one or more of the following structural factors: the presence of electron withdrawing groups which stabilize the thiolate through sigma and pi bonds (inductive effect), the presence of electron withdrawing groups that stabilize the thiolate through space or solvent (field effects), pi bonds which allow the negative charge to be placed on other atoms (resonance stabilization), and hydrogen bond donating groups within the molecule that can interact internally with the thiolate. For example, cysteine has an amino group two atoms from the thiol, which is more electron withdrawing than the amide nitrogen that is two atoms from the thiol in glutathione. As a consequence of this difference in electron withdrawing groups, the thiol of cysteine is 0.6 pK units more acidic than glutathione, and as mentioned previously, cystine is reduced 3-15 times faster than oxidized glutathione. Another example of a relatively acidic thiol is 5-thio-2-nitrobenzoic acid, pK_a 5. Its acidity is due to resonance stabilization and inductive effects. Its disulfide is rapidly reduced by all standard alkyl thiols and its colored thiolate makes it a convenient assay for thiol concentration.

Summary

Described in a preferred embodiment is a process for the delivery of a compound to a cell, comprising associating a compound, containing a disulfide bond that can be cleaved under physiological conditions, with a polymer, then delivering the polymer to the cell. The polymer may comprise a first polymer and a second polymer. The first

polymer and the second polymer may comprise nucleic acids, proteins, genes, antisense polymers, DNA/RNA hybrids, or synthetic polymers.

In another preferred embodiment, a biologically active compound is associated with a disulfide-containing compound, comprising: the disulfide-containing compound having a labile disulfide bond that is selected from the group consisting of (a) a disulfide bond that is cleaved more rapidly than oxidized glutathione and (b) a disulfide bond constructed from thiols in which one of the constituent thiols has a lower pKa than glutathione and (c) a disulfide bond that is activated by intramolecular attack from a free thiol.

In another preferred embodiment, a compound is provided for inserting into an organism, comprising: the compound having a disulfide bond that is labile under physiologic conditions selected from the group consisting of (a) a disulfide bond that is cleaved more rapidly than oxidized glutathione and (b) a disulfide bond constructed from thiols in which one of the constituent thiols has a lower pKa than glutathione and (c) a disulfide bond that is activated by intramolecular attack from a free thiol.

In another preferred embodiment, a process is provided for forming a compound having a labile disulfide bond for use with an organism, comprising: forming the compound having a disulfide bond selected from the group consisting of (i) a disulfide bond that is cleaved more rapidly than oxidized glutathione, and (ii) a disulfide bond constructed from thiols in which one of the constituent thiols has a lower pKa than glutathione, and (iii) a disulfide bond that is activated by intramolecular attack from a free thiol:
inserting the compound into the organism.

In another preferred embodiment, a process is described for compacting a nucleic acid for delivery to a cell, comprising associating a polymer containing a disulfide bond with a nucleic acid and delivering the nucleic acid to the cell.

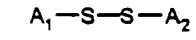
In another preferred embodiment, a process is described for compacting a nucleic acid for delivery to a cell comprising associating a polymer with the nucleic acid, then associating a compound containing a disulfide bond that can be cleaved under

physiological conditions with the nucleic acid polymer complex, then delivering the complex to a cell.

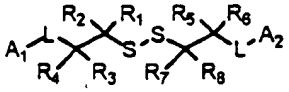
In another preferred embodiment, a process is described for compacting a nucleic acid for delivery to a cell, comprising associating a polymer containing a disulfide bond with a nucleic acid, then associating another polymer with the disulfide containing polymer – nucleic acid complex, then delivering the complex to the cell.

In another preferred embodiment, a process is described for compacting a nucleic acid for delivery to a cell comprising associating a polymer with the nucleic acid, then associating a compound containing a disulfide bond that can be cleaved under physiological conditions with the nucleic acid polymer complex, then associating another polymer with the complex, then delivering the complex to a cell.

In another preferred embodiment, a compound is described which contains a disulfide bond that can be cleaved under physiological conditions and possesses heterobifunctional or homobifunctional groups. Such a compound can be described as a disulfide containing bifunctional molecule.



More particularly, a compound that contains an aliphatic disulfide bond with one or more electronegative (electron withdrawing groups) substituted alpha or beta to one or both of the sulfur atoms. These groups serve to lower the pK_a of the constituent thiols.



Where R_1, R_2, R_3, R_4, R_5, R_6, R_7, R_8 - at least one of which is an electronegative atom or functionality such as OH, OR (an ether), NH_2 (also secondary, tertiary, and quaternary amines), SO_3^-, COOH, COOR (an ester), $CONH_2$, $CONR_2$ (substituted amide), a halogen (F, Cl, Br, I), NO_2. L is defined as a linker or spacer group that

provides a connection between the disulfide and the reactive heterobifunctional or homobifunctional groups, A_1 and A_2. L may or may not be present and may be chosen from a group that includes alkanes, alkenes, alkynes, esters, ethers, glycerol, amide, urea, saccharides, polysaccharides, heteroatoms such as oxygen, sulfur, or nitrogen. The spacer may be charge positive, charge negative, charge neutral, or zwitterionic. A_1 and A_2 are reactive groups they may be identical as in a homobifunctional bifunctional molecule, or different as in a heterobifunctional bifunctional molecule. In a preferred embodiment, the disulfide compounds contain reactive groups that can undergo acylation or alkylation reactions. Such reactive groups include (but not limited to) isothiocyanate, isocyanate, acyl azide, acid halide, O-acyl urea, N-hydroxysuccinimide esters, succinimide esters, amide, urea, sulfonyl chloride, aldehyde, ketone, ether, epoxide, carbonate, alkyl halide, imidoester, carboxylate, alkylphosphate, arylhalides (e.g. difluoro-dinitrobenzene) or anhydrides.

If functional group A1,A2 is an amine then A1,A2 can react with (but not restricted to) an activated carboxylic acid, isothiocyanate, isocyanate, acyl azide, alkyl halide, acid halide, N-hydroxysuccinimide ester, sulfonyl chloride, aldehyde, ketone, epoxide, carbonate, imidoester, amide, carboxylate, or alkylphosphate, arylhalides (difluoro-dinitrobenzene) or anhydrides. In other terms when function A1,A2 is an amine, then an acylating or alkylating agent can react with the amine.

If functional group A1,A2 is a sulfhydryl then A1,A2 can react with (but not restricted to) a haloacetyl derivative, activated carboxylic acid, maleimide, aziridine derivative, acryloyl derivative, fluorobenzene derivatives, or disulfide derivative (such as a pyridyl disulfide or 5-thio-2-nitrobenzoic acid{TNB} derivatives).

If functional group A1,A2 is carboxylate then A1,A2 can react with (but not restricted to) a diazoacetate, alcohol, thiol or an amine once the acid has been activated.

If functional group A1,A2 is an hydroxyl then A1,A2 can react with (but not restricted to) an activated carboxylic acid, epoxide, oxirane, or an amine in which carbonyldiimidazole is used.

If functional group A1,A2 is an aldehyde or ketone then A1,A2 can react with (but not restricted to) an hydrazine, hydrazide derivative, amine (to form a Schiff Base that may or may not be subsequently reduced by reducing agents such as $NaCNBH_3$), or a diol to form an acetal or ketal.

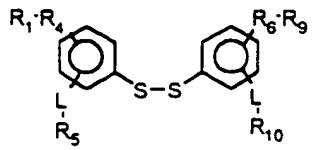
If functional group A1,A2 is activated carboxylic acid, isothiocyanate, isocyanate, acyl azide, N-hydroxysuccinimide ester, sulfonyl chloride, aldehyde, ketone, epoxide, carbonate, imidoester, alkylphosphate, arylhalides (difluoro-dinitrobenzene), anhydride, alkyl halide, or acid halide, p-nitrophenyl ester, o-nitrophenyl ester, pentachlorophenyl ester, pentafluorophenyl ester, carbonyl imidazole, carbonyl pyridinium, or carbonyl dimethylaminopyridinium, then A1,A2 can react with (but not restricted to) an amine, a hydroxyl, hydrazine, hydrazide, or sulfhydryl group.

If functional group A1,A2 an activated carboxylic acid, haloacetyl derivative, maleimide, aziridine derivative, acryloyl derivative, fluorobenzene derivatives, or disulfide derivative (such as a pyridyl disulfide or 5-thio-2-nitrobenzoic acid{TNB} derivatives) then A1,A2 can react with (but not restricted to) a sulfhydryl.

If functional group A1,A2 is an aldehyde, ketone, epoxide, oxirane, or an amine in which carbonyldiimidazole or N, N'-disuccinimidyl carbonate is used, then A1,A2 can react with (but not restricted to) a hydroxyl.

If functional group A1,A2 is a hydrazine, hydrazide derivative, or amine (primary or secondary) then A1,A2 can react with (but not restricted to) an aldehyde or ketone (to form a Schiff Base that may or may not be reduced by reducing agents such as $NaCNBH_3$).

Additionally, a compound which contains an aromatic disulfide bond in which the sulfur atom is bonded directly to the aromatic ring. The ring may contain 5 or more atoms.



R_1-R_4, R_6-R_9 - The substitution pattern on the ring may be varied to alter the reduction potential of the disulfide bond. The substiuents may be selected from the group that includes but is not limited to OH, OR (an ether), NH_2,(also secondary, tertiary, and quaternary amines), SO_3^-, COOH, COOR (an ester), $CONH_2$, $CONR_2$ (substituted amide), a halogen (F, Cl, Br, I), NO_2, CH_3 (or longer branched or straight chain, saturated, or unsaturated aliphatic group). L is defined as a linker or spacer group that provides a connection between the disulfide and the reactive heterobifunctional or homobifunctional groups. L may or may not be present and may be chosen from a group that includes alkanes, alkenes, esters, ethers, glycerol, amide, saccharides, polysaccharides, heteroatoms such as oxygen, sulfur, or nitrogen. The spacer may be charge positive, charge negative, charge neutral, or zwitterionic. R_5, R_{10} - are reactive groups they may be identical as in a homobifunctional bifunctional molecule, or different as in a heterobifunctional bifunctional molecule. In a preferred embodiment, the disulfide compounds contain reactive groups that can undergo acylation or alkylation reactions. Such reactive groups include isothiocynanate, isocynanate, acyl azide, N-hydroxysuccinimide esters, succinimide esters, sulfonyl chloride, aldehyde, epoxide, carbonate, imidoester, carboxylate, alkylphosphate, arylhalides (e.g. difluoro-dinitrobenzene) or succinic anhydride.

If functional group R5, R10 is an amine then R5, R10 can react with (but not restricted to) an activated carboxylic acid, isothiocyanate, isocyanate, acyl azide, alkyl halide, acid halide, N-hydroxysuccinimide ester, sulfonyl chloride, aldehyde, ketone, epoxide, carbonate, imidoester, amide, carboxylate, or alkylphosphate, arylhalides (difluoro-dinitrobenzene) or anhydrides. In other terms when function R5, R10 is an amine, then an acylating or alkylating agent can react with the amine.

If functional group R5, R10 is a sulfhydryl then R5, R10 can react with (but not restricted to) a haloacetyl derivative, activated carboxylic acid, maleimide, aziridine

derivative, acryloyl derivative, fluorobenzene derivatives, or disulfide derivative (such as a pyridyl disulfide or 5-thio-2-nitrobenzoic acid{TNB} derivatives).

If functional group R5, R10 is carboxylate then R5, R10 can react with (but not restricted to) a diazoacetate, alcohol, thiol or an amine once the acid has been activated.

If functional group R5, R10 is an hydroxyl then R5, R10 can react with (but not restricted to) an activated carboxylic acid, epoxide, oxirane, or an amine in which carbonyldiimidazole is used.

If functional group R5, R10 is an aldehyde or ketone then R5, R10 can react with (but not restricted to) an hydrazine, hydrazide derivative, amine (to form a Schiff Base that may or may not be subsequently reduced by reducing agents such as $NaCNBH_3$), or a diol to form an acetal or ketal.

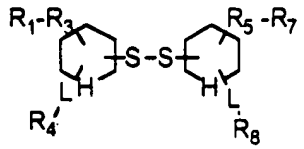
If functional group R5, R10 is activated carboxylic acid, isothiocyanate, isocyanate, acyl azide, N-hydroxysuccinimide ester, sulfonyl chloride, aldehyde, ketone, epoxide, carbonate, imidoester, alkylphosphate, arylhalides (difluoro-dinitrobenzene), anhydride, alkyl halide, or acid halide, p-nitrophenyl ester, o-nitrophenyl ester, pentachlorophenyl ester, pentafluorophenyl ester, carbonyl imidazole, carbonyl pyridinium, or carbonyl dimethylaminopyridinium then R5, R10 can react with (but not restricted to) an amine, a hydroxyl, hydrazine, hydrazide, or sulfhydryl group.

If functional group R5, R10 an activated carboxylic acid, haloacetyl derivative, maleimide, aziridine derivative, acryloyl derivative, fluorobenzene derivatives, or disulfide derivative (such as a pyridyl disulfide or 5-thio-2-nitrobenzoic acid{TNB} derivatives) then R5, R10 can react with (but not restricted to) a sulfhydryl.

If functional group R5, R10 is an aldehyde, ketone, epoxide, oxirane, or an amine in which carbonyldiimidazole or N, N'-disuccinimidyl carbonate is used, then R5, R10 can react with (but not restricted to) a hydroxyl.

If functional group R5. R10 is a hydrazine, hydrazide derivative, or amine (primary or secondary) then R5. R10 can react with (but not restricted to) an aldehyde or ketone (to form a Schiff Base that may or may not be reduced by reducing agents such as $NaCNBH_3$).

Additionally, a compound which contains a disulfide bond that is connected directly to a heterocyclic ring. The heterocyclic ring may be aromatic or aliphatic. The heterocyclic ring may contain 5 or more atoms of which 1 or more is a heteroatom (O, N, S, P), and the rest being carbon atoms



H is a heteroatom selected from the group including sulfur, oxygen, nitrogen, or phosphorus. R_1-R_3, R_5-R_7 are substiuents that may be selected from the group that includes but is not limited to OH, OR (an ether), NH_2,(also secondary, tertiary, and quaternary amines), SO_3^-, COQH, COOR (an ester), $CONH_2$, $CONR_2$ (substituted amide), a halogen (F, Cl, Br, I), NO_2, CH_3 (or longer branched or straight chain, saturated, or unsaturated aliphatic group). The substitution pattern on the aromatic ring may be varied to alter the reduction potential of the disulfide bond. L is defined as a linker or spacer group that provides a connection between the disulfide and the reactive heterobifunctional or homobifunctional groups. L may or may not be present and may be chosen from a group that includes alkanes, alkenes, esters, ethers, glycerol, amide, saccharides, polysaccharides, heteroatoms such as oxygen, sulfur, or nitrogen. The spacer may be charge positive, charge negative, charge neutral, or zwitterionic. R_4, R_8 are reactive groups they may be identical as in a homobifunctional bifunctional molecule, or different as in a heterobifunctional bifunctional molecule. In a preferred embodiment, the disulfide compounds contain reactive groups that can undergo acylation or alkylation reactions. Such reactive groups include isothiocynanate, isocynanate, acyl azide, N-hydroxysuccinimide esters, succinimide esters, sulfonyl chloride, aldehyde, epoxide, carbonate, imidoester,

carboxylate, alkylphosphate. arylhalides (e.g. difluoro-dinitrobenzene) or succinic anhydride.

If functional group R4. R8 is an amine then R4. R8 can react with (but not restricted to) an activated carboxylic acid, isothiocyanate, isocyanate, acyl azide, alkyl halide, acid halide, N-hydroxysuccinimide ester, sulfonyl chloride, aldehyde, ketone, epoxide, carbonate, imidoester, amide, carboxylate, or alkylphosphate, arylhalides (difluoro-dinitrobenzene) or anhydrides. In other terms when function R4, R8 is an amine, then an acylating or alkylating agent can react with the amine.

If functional group R4, R8 is a sulfhydryl then R4, R8 can react with (but not restricted to) a haloacetyl derivative, activated carboxylic acid, maleimide, aziridine derivative, acryloyl derivative, fluorobenzene derivatives, or disulfide derivative (such as a pyridyl disulfide or 5-thio-2-nitrobenzoic acid{TNB} derivatives).

If functional group R4, R8 is carboxylate then R4, R8 can react with (but not restricted to) a diazoacetate, alcohol, thiol or an amine once the acid has been activated.

If functional group R4, R8 is an hydroxyl then R4. R8 can react with (but not restricted to) an activated carboxylic acid, epoxide, oxirane, or an amine in which carbonyldiimidazole is used.

If functional group R4. R8 is an aldehyde or ketone then R4. R8 can react with (but not restricted to) an hydrazine, hydrazide derivative. amine (to form a Schiff Base that may or may not be subsequently reduced by reducing agents such as $NaCNBH_3$), or a diol to form an acetal or ketal.

If functional group R4, R8 is activated carboxylic acid, isothiocyanate, isocyanate, acyl azide, N-hydroxysuccinimide ester, sulfonyl chloride, aldehyde, ketone, epoxide, carbonate, imidoester. alkylphosphate. arylhalides (difluoro-dinitrobenzene), anhydride, alkyl halide. or acid halide, p-nitrophenyl ester, o-nitrophenyl ester, pentachlorophenyl ester. pentafluorophenyl ester, carbonyl imidazole, carbonyl

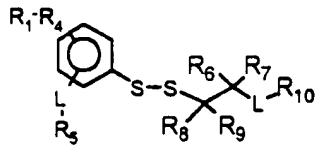
pyridinium, or carbonyl dimethylaminopyridinium, then R4, R8 can react with (but not restricted to) an amine, a hydroxyl, hydrazine, hydrazide, or sulfhydryl group.

If functional group R4, R8 an activated carboxylic acid, haloacetyl derivative, maleimide, aziridine derivative, acryloyl derivative, fluorobenzene derivatives, or disulfide derivative (such as a pyridyl disulfide or 5-thio-2-nitrobenzoic acid{TNB} derivatives) then R4, R8 can react with (but not restricted to) a sulfhydryl.

If functional group R4, R8 is an aldehyde, ketone, epoxide, oxirane, or an amine in which carbonyldiimidazole or N, N'-disuccinimidyl carbonate is used, then R4, R8 can react with (but not restricted to) a hydroxyl.

If functional group R4, R8 is a hydrazine, hydrazide derivative, or amine (primary or secondary) then R4, R8 can react with (but not restricted to) an aldehyde or ketone (to form a Schiff Base that may or may not be reduced by reducing agents such as $NaCNBH_3$).

Additionally, a compound which contains a disulfide bond that is connected directly to a ring system(aromatic or non-aromatic) through one of the sulfur atoms and to a aliphatic carbon through the other sulfur atom. The cyclic ring may contain 5 or more atoms.



R_1-R_4 are substiuents selected from the group that includes but is not limited to H, OH, OR (an ether), NH_2(also secondary, tertiary, and quaternary amines), SO_3^-, COOH, COOR (an ester), $CONH_2$, $CONR_2$ (substituted amide), a halogen (F, Cl, Br, I), NO_2, CH_3 (or longer branched or straight chain, saturated, or unsaturated aliphatic group). The substitution pattern on the aromatic ring may be varied to alter the reduction potential of the disulfide bond. R_6-R_9 are substiuents selected from the group that includes but is not limited to H, OH, OR (an ether), NH_2(also secondary,

tertiary, and quaternary amines), SO_3^-, COOH, COOR (an ester), $CONH_2$, $CONR_2$ (substituted amide), a halogen (F, Cl, Br, I), NO_2, CH_3 (or longer branched or straight chain, saturated, or unsaturated aliphatic group). L is defined as a linker or spacer group that provides a connection between the disulfide and the reactive heterobifunctional or homobifunctional groups. L may or may not be present and may be chosen from a group that includes alkanes, alkenes, esters, ethers, glycerol, amide, saccharides, polysaccharides, heteroatoms such as oxygen, sulfur, or nitrogen. The spacer may be charge positive, charge negative, charge neutral, or zwitterionic. R_5, and R_{10} are reactive groups that may be identical as in a homobifunctional bifunctional molecule, or different as in a heterobifunctional bifunctional molecule. In a preferred embodiment, the disulfide compounds contain reactive groups that can undergo acylation or alkylation reactions. Such reactive groups include isothiocynanate, isocynanate, acyl azide, N-hydroxysuccinimide esters, succinimide esters, sulfonyl chloride, aldehyde, epoxide, carbonate, imidoester, carboxylate, alkylphosphate, arylhalides (e.g. difluoro-dinitrobenzene) or succinic anhydride.

If functional group R5, R10 is an amine then R5, R10 can react with (but not restricted to) an activated carboxylic acid, isothiocyanate, isocyanate, acyl azide, alkyl halide, acid halide. N-hydroxysuccinimide ester, sulfonyl chloride, aldehyde, ketone, epoxide, carbonate, imidoester, amide, carboxylate, or alkylphosphate, arylhalides (difluoro-dinitrobenzene) or anhydrides. In other terms when function R5, R10 is an amine, then an acylating or alkylating agent can react with the amine.

If functional group R5, R10 is a sulfhydryl then R5, R10 can react with (but not restricted to) a haloacetyl derivative, activated carboxylic acid, maleimide, aziridine derivative, acryloyl derivative, fluorobenzene derivatives, or disulfide derivative (such as a pyridyl disulfide or 5-thio-2-nitrobenzoic acid{TNB} derivatives).

If functional group R5, R10 is carboxylate then R5, R10 can react with (but not restricted to) a diazoacetate, alcohol, thiol or an amine once the acid has been activated.

If functional group R5, R10 is an hydroxyl then R5, R10 can react with (but not restricted to) an activated carboxylic acid, epoxide, oxirane, or an amine in which carbonyldiimidazole is used.

If functional group R5, R10 is an aldehyde or ketone then R5, R10 can react with (but not restricted to) an hydrazine, hydrazide derivative, amine (to form a Schiff Base that may or may not be subsequently reduced by reducing agents such as $NaCNBH_3$), or a diol to form an acetal or ketal.

If functional group R5, R10 is activated carboxylic acid, isothiocyanate, isocyanate, acyl azide, N-hydroxysuccinimide ester, sulfonyl chloride, aldehyde, ketone, epoxide, carbonate, imidoester, alkylphosphate, arylhalides (difluoro-dinitrobenzene), anhydride, alkyl halide, or acid halide, p-nitrophenyl ester, o-nitrophenyl ester, pentachlorophenyl ester, pentafluorophenyl ester, carbonyl imidazole, carbonyl pyridinium, or carbonyl dimethylaminopyridinium, then R5, R10 can react with (but not restricted to) an amine, a hydroxyl, hydrazine, hydrazide, or sulfhydryl group.

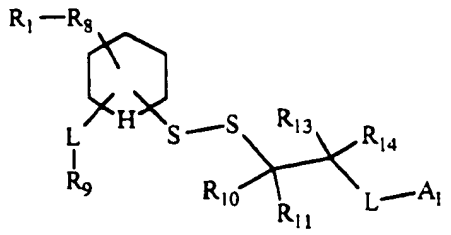
If functional group R5, R10 an activated carboxylic acid, haloacetyl derivative, maleimide, aziridine derivative, acryloyl derivative, fluorobenzene derivatives, or disulfide derivative (such as a pyridyl disulfide or 5-thio-2-nitrobenzoic acid{TNB} derivatives) then R5, R10 can react with (but not restricted to) a sulfhydryl.

If functional group R5, R10 is an aldehyde, ketone, epoxide, oxirane, or an amine in which carbonyldiimidazole or N, N'-disuccinimidyl carbonate is used, then R5, R10 can react with (but not restricted to) a hydroxyl.

If functional group R5, R10 is a hydrazine, hydrazide derivative, or amine (primary or secondary) then R5, R10 can react with (but not restricted to) an aldehyde or ketone (to form a Schiff Base that may or may not be reduced by reducing agents such as $NaCNBH_3$).

Additionally, a compound which contains a disulfide bond that is connected directly to a heterocyclic ring system through one of the sulfur atoms and to a aliphatic carbon

through the other sulfur atom. The heterocyclic ring may contain 5 or more atoms of which 1 or more is a heteroatom (O, N, S, P) or combinations of heteroatoms, and the rest being carbon atoms.



H is a heteroatom selected from the group including sulfur, oxygen, nitrogen, or phosphorus. Where R_1, R_2, R_3, R_4, R_5, R_6, R_7, R_8 ,R_9, R10, R11, R12, R14- at least one of which is an electronegative atom or functionality such as OH, OR (an ether), . NH_2(also secondary, tertiary, and quaternary amines), SO_3^-, COOH, COOR (an ester), $CONH_2$, $CONR_2$ (substituted amide), a halogen (F, Cl, Br, I), NO_2. L is defined as a linker or spacer group that provides a connection between the disulfide and the reactive heterobifunctional or homobifunctional groups, A_1 and R9. L may or may not be present and may be chosen from a group that includes alkanes, alkenes, alkynes, esters, ethers, glycerol, amide, urea, saccharides, polysaccharides, heteroatoms such as oxygen, sulfur, or nitrogen. The spacer may be charge positive, charge negative, charge neutral, or zwitterionic. A_1 and R9 are reactive groups they may be identical as in a homobifunctional bifunctional molecule, or different as in a heterobifunctional bifunctional molecule. In a preferred embodiment, the disulfide compounds contain reactive groups that can undergo acylation or alkylation reactions. Such reactive groups include (but not limited to) isothiocynanate, isocynanate, acyl azide, acid halide, O-acyl urea, N-hydroxysuccinimide esters, succinimide esters, amide, urea, sulfonyl chloride, aldehyde, ketone, ether, epoxide, carbonate, alkyl halide, imidoester, carboxylate, alkylphosphate, arylhalides (e.g. difluoro-dinitrobenzene) or anhydrides.

If functional group A1,R9 is an amine then A1,R9 can react with (but not restricted to) an activated carboxylic acid, isothiocyanate, isocyanate, acyl azide, alkyl halide, acid halide, N-hydroxysuccinimide ester, sulfonyl chloride, aldehyde, ketone, epoxide, carbonate, imidoester, amide, carboxylate, or alkylphosphate, arylhalides

(difluoro-dinitrobenzene) or anhydrides. In other terms when function A1,R9 is an amine, then an acylating or alkylating agent can react with the amine.

If functional group A1,R9 is a sulfhydryl then A1,R9 can react with (but not restricted to) a haloacetyl derivative, activated carboxylic acid, maleimide, aziridine derivative, acryloyl derivative, fluorobenzene derivatives, or disulfide derivative (such as a pyridyl disulfide or 5-thio-2-nitrobenzoic acid{TNB} derivatives).

If functional group A1,R9 is carboxylate then A1,R9 can react with (but not restricted to) a diazoacetate, alcohol, thiol or an amine once the acid has been activated.

If functional group A1,R9 is an hydroxyl then A1,R9 can react with (but not restricted to) an activated carboxylic acid, epoxide, oxirane, or an amine in which carbonyldiimidazole is used.

If functional group A1,R9 is an aldehyde or ketone then A1,R9 can react with (but not restricted to) an hydrazine, hydrazide derivative, amine (to form a Schiff Base that may or may not be subsequently reduced by reducing agents such as $NaCNBH_3$), or a diol to form an acetal or ketal.

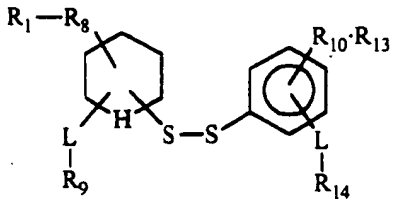
If functional group A1,R9 is activated carboxylic acid, isothiocyanate, isocyanate, acyl azide, N-hydroxysuccinimide ester, sulfonyl chloride, aldehyde, ketone, epoxide, carbonate, imidoester, alkylphosphate, arylhalides (difluoro-dinitrobenzene), anhydride, alkyl halide, or acid halide, p-nitrophenyl ester, o-nitrophenyl ester, pentachlorophenyl ester, pentafluorophenyl ester, carbonyl imidazole, carbonyl pyridinium, or carbonyl dimethylaminopyridinium, then A1,R9 can react with (but not restricted to) an amine, a hydroxyl, hydrazine, hydrazide, or sulfhydryl group.

If functional group A1,R9 an activated carboxylic acid, haloacetyl derivative, maleimide, aziridine derivative, acryloyl derivative, fluorobenzene derivatives, or disulfide derivative (such as a pyridyl disulfide or 5-thio-2-nitrobenzoic acid{TNB} derivatives) then A1,R9 can react with (but not restricted to) a sulfhydryl.

If functional group A1,R9 is an aldehyde, ketone, epoxide, oxirane, or an amine in which carbonyldiimidazole or N, N'-disuccinimidyl carbonate is used, then A1,R9 can react with (but not restricted to) a hydroxyl.

If functional group A1,R9 is a hydrazine, hydrazide derivative, or amine (primary or secondary) then A1,R9 can react with (but not restricted to) an aldehyde or ketone (to form a Schiff Base that may or may not be reduced by reducing agents such as $NaCNBH_3$).

Additionally, a compound which contains a disulfide bond that is connected directly to a heterocyclic ring system (aromatic or non-aromatic) through one of the sulfur atoms and to an aromatic ring system through the other sulfur atom. The heterocyclic ring may contain 5 or more atoms of which 1 or more is a heteroatom (O, N, S, P) or combinations of heteroatoms, and the rest being carbon atoms.



H is a heteroatom selected from the group including sulfur, oxygen, nitrogen, or phosphorus. Where R_1, R_2, R_3, R_4, R_5, R_6, R_7, R_8, R_{10}, R_{11}, R_{12}, R_{13}- at least one of which is an electronegative atom or functionality such as OH, OR (an ether), NH_2(also secondary, tertiary, and quaternary amines), SO_3^-, COOH, COOR (an ester), $CONH_2$, $CONR_2$ (substituted amide), a halogen (F, Cl, Br, I), NO_2. L is defined as a linker or spacer group that provides a connection between the disulfide and the reactive heterobifunctional or homobifunctional groups, R_9 and R_{14}. L may or may not be present and may be chosen from a group that includes alkanes, alkenes, alkynes, esters, ethers, glycerol, amide, urea, saccharides, polysaccharides, heteroatoms such as oxygen, sulfur, or nitrogen. The spacer may be charge positive, charge negative, charge neutral, or zwitterionic. R_9 and R_{14} are reactive groups they may be identical as in a homobifunctional bifunctional molecule, or different as in a heterobifunctional bifunctional molecule. In a preferred embodiment, the disulfide compounds contain reactive groups that can undergo acylation or alkylation reactions.

Such reactive groups include (but not limited to) isothiocynanate, isocynanate, acyl azide, acid halide. O-acyl urea, N-hydroxysuccinimide esters, succinimide esters, amide, urea, sulfonyl chloride, aldehyde, ketone, ether, epoxide, carbonate, alkyl halide, imidoester, carboxylate, alkylphosphate, arylhalides (e.g. difluoro-dinitrobenzene) or anhydrides.

If functional group R9,R14 is an amine then R9,R14 can react with (but not restricted to) an activated carboxylic acid, isothiocyanate, isocyanate, acyl azide, alkyl halide, acid halide, N-hydroxysuccinimide ester, sulfonyl chloride, aldehyde, ketone, epoxide, carbonate. imidoester, amide, carboxylate, or alkylphosphate, arylhalides (difluoro-dinitrobenzene) or anhydrides. In other terms when function R9,R14 is an amine, then an acylating or alkylating agent can react with the amine.

If functional group R9,R14 is a sulfhydryl then R9,R14 can react with (but not restricted to) a haloacetyl derivative, activated carboxylic acid, maleimide, aziridine derivative, acryloyl derivative, fluorobenzene derivatives, or disulfide derivative (such as a pyridyl disulfide or 5-thio-2-nitrobenzoic acid{TNB} derivatives).

If functional group R9,R14 is carboxylate then R9,R14 can react with (but not restricted to) a diazoacetate, alcohol, thiol or an amine once the acid has been activated.

If functional group R9,R14 is an hydroxyl then R9,R14 can react with (but not restricted to) an activated carboxylic acid, epoxide, oxirane, or an amine in which carbonyldiimidazole is used.

If functional group R9,R14 is an aldehyde or ketone then R9,R14 can react with (but not restricted to) an hydrazine, hydrazide derivative, amine (to form a Schiff Base that may or may not be subsequently reduced by reducing agents such as $NaCNBH_3$), or a diol to form an acetal or ketal.

If functional group R9,R14 is activated carboxylic acid, isothiocyanate, isocyanate, acyl azide. N-hydroxysuccinimide ester. sulfonyl chloride, aldehyde, ketone. epoxide,

carbonate, imidoester, alkylphosphate, arylhalides (difluoro-dinitrobenzene), anhydride, alkyl halide, or acid halide, p-nitrophenyl ester, o-nitrophenyl ester, pentachlorophenyl ester, pentafluorophenyl ester, carbonyl imidazole, carbonyl pyridinium, or carbonyl dimethylaminopyridinium, then R9,R14 can react with (but not restricted to) an amine, a hydroxyl, hydrazine, hydrazide, or sulfhydryl group.

If functional group R9,R14 an activated carboxylic acid, haloacetyl derivative, maleimide, aziridine derivative, acryloyl derivative, fluorobenzene derivatives, or disulfide derivative (such as a pyridyl disulfide or 5-thio-2-nitrobenzoic acid{TNB} derivatives) then R9,R14 can react with (but not restricted to) a sulfhydryl.

If functional group R9,R14 is an aldehyde, ketone, epoxide, oxirane, or an amine in which carbonyldiimidazole or N, N'-disuccinimidyl carbonate is used, then R9,R14 can react with (but not restricted to) a hydroxyl.

If functional group R9,R14 is a hydrazine, hydrazide derivative, or amine (primary or secondary) then R9,R14 can react with (but not restricted to) an aldehyde or ketone (to form a Schiff Base that may or may not be reduced by reducing agents such as $NaCNBH_3$).

Detailed Description

Counterintuitive to previous efforts to synthesize bifunctional molecules with stabile disulfides, the object of the current invention is to synthesize labile disulfide molecules. In vivo, disulfides are primarily reduced by the cysteine-based thiol glutathione (γ-glutamylcystylglycine), which is present in millimolar concentrations in the cell. To increase the lability of the disulfide bond in a bifunctional molecule and its construct, we have synthesized several disulfide bond-containing bifunctional molecules that are more rapidly reduced than oxidized glutathione.

Disulfide Bond Containing Bifunctional molecules

Bifunctional molecules, possessing either homo or heterobifunctionality (commonly referred to as crosslinkers), are used to connect two molecules together. The disulfide linkage (RSSR') may be used within bifunctional molecules. The reversibility of

We Claim:

1) A biologically active compound associated with a disulfide-containing compound, comprising: the disulfide-containing compound having a labile disulfide bond that is selected from the group consisting of (a) a disulfide bond that is cleaved more rapidly than oxidized glutathione and (b) a disulfide bond constructed from thiols in which one of the constituent thiols has a lower pKa than glutathione and (c) a disulfide bond that is activated by intramolecular attack from a free thiol.

2) The compounds of claim 1 wherein the disulfide-containing compound comprises a polymer.

3) The compound of claim 2 wherein the polymer is selected from the group consisting of a polycation, a polyanion, a neutral polymer and an amphipathic polymer.

4) The compound of claim 1 wherein the biologically active compound is a polynucleotide.

5) The compound of claim 1 wherein the biologically active compound is a polypeptide.

6) The compound of claim 1 wherein the disulfide-containing compound contains a ligand.

7) A compound for inserting into an organism, comprising: the compound having a disulfide bond that is labile under physiologic conditions selected from the group consisting of (a) a disulfide bond that is cleaved more rapidly than oxidized glutathione and (b) a disulfide bond constructed from thiols in which one of the constituent thiols has a lower pKa than glutathione and (c) a disulfide bond that is activated by intramolecular attack from a free thiol.

8) The compound of claim 7 wherein the compound comprises an amphipathic compound.

9) The compound of claim 7 wherein the compound comprises a polymer.

10) The method of claim 7 wherein the polymer is selected from the group consisting of a polycation, a polyanion, a neutral polymer, and an amphipathic polymer.

11) The method of claim 7 wherein the compound contains a ligand.

12) A process for forming a compound having a labile disulfide bond for use with an organism, comprising:

a) forming the compound having a disulfide bond selected from the group consisting of (i) a disulfide bond that is cleaved more rapidly than oxidized glutathione, and (ii) a disulfide bond constructed from thiols in which one of the constituent thiols has a lower pKa than glutathione, and (iii) a disulfide bond that is activated by intramolecular attack from a free thiol;

b) inserting the compound into the organism.

13) The process of claim 12 wherein the compound comprises a polymer.

14) The process of claim 12 wherein the polymer is selected from the group consisting of a polycation, a polyanion, a neutral polymer, and an amphipathic polymer.

15) The process of claim 12 wherein the compound having a labile disulfide bond is associated with a biologically active compound.

16) The process of claim 15 wherein the biologically active compound is a polynucleotide.

17) The process of claim 15 wherein the biologically active compound is a polypeptide.

18) The process of claim 12 wherein the disulfide is a bifunctional molecule.